Voluntary - Public

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National Food Safety Standard - Choline

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FAIRS Subject Report

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Report Highlights:

On November 20, 2009, China notified the WTO of "National Food Safety Standard of the People's Republic of China for Determination of Choline in Foods for Infants and Young Children, Raw Milk, and Dairy Products" as SPS/N/CHN/164. The date for submission of final comments to the WTO is January 1, 2010. The proposed date of entry into force has not been specified.

Executive Summary:

On November 20, 2009, China notified the WTO of "National Food Safety Standard of the People's Republic of China for Determination of Choline in Foods for Infants and Young Children, Raw Milk, and Dairy Products" as SPS/N/CHN/164. The date for submission of final comments to the WTO is January 1, 2010. The proposed date of entry into force has not been specified.

Thanks go to the consortium of industry and 3rd country Embassies in Beijing for their assistance in translating and reviewing this standard.

This report contains an UNOFFICIAL translation of National Food Safety Standard of the People's Republic of China for Determination of Choline in Foods for Infants and Young Children, Raw Milk, and Dairy Products.

General Information:

BEGIN TRANSLATION

ICS 67.100.10

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National Standard for Food Safety of the People's Republic of China

GB ××××—××××

Substituting GB/T 5413.20—1997

Determination of Choline in Foods for Infants and Young Children, Raw Milk, and Dairy Products

(Draft for comments)

Promulgated on ××-××-××××

Takes effect as of ××-××-××××

Promulgated by the Ministry of Health

of the People's Republic of China

Foreword

This standard supersedes GB/T 5413.20-1997 Determination of Choline in Formulated Foods for Infants and Young Children and Milk Powder.

Compared with GB/T 5413-23-1997, this standard:

— has changed the ingredients of color developing agent for enzyme reaction

— includes the second method: Reinecke's salt spectrophotometric method and the third method: ion chromatographic method

Appendix A attached to this standard is an informative annex.

This standard is proposed by and put under centralized management by the Ministry of Health of the People's Republic of China

Previous standard editions substituted by this edition are as follows:

— GB 5413 – 1985; GB/T 5413.23 – 1997

Determination of Choline in Foods for Infants and Young Children, Raw Milk, and Dairy Products

1. Scope

This standard outlines the method of determination of choline in foods for infants and young children, raw milk, and dairy products.

The first method and second method in this standard are applicable to the determination of choline in foods for infants and young children, raw milk, and dairy products; the third method in this standard is applicable to determination of choline in foods for infants and young children and milk powder.

The detection limit for the first method in this standard is 20mg/kg; that for the second method is 50mg/kg; and that for the third method is 2mg/kg.

2. Referenced regulatory documents

Clauses in the following documents become those of this standard by reference in this standard. As regards referenced documents with indicated dates, none of the amendments (not including the content of corrigendum) made to them after these dates is applicable to this standard; however, parties to an agreement concluded according to this standard are encouraged to consider whether they will adopt the latest edition of these documents or not. Any latest edition of any referenced document without indicated date is applicable to this standard.

GB/T6682 Water Specification and Test Method for Analytical Laboratory

The First Method: Enzymatic Colorimetric Method

3. Principle

Chlorine in the test samples is turned into free choline, and then it is oxidized with enzyme and reacts with developing agent to generate color matter, whose shade is positively proportional to choline content in a certain range.

4. Reagents and materials

Unless otherwise provided, in this method, all reagents are analytically pure and the water is third level water as provided in GB/T6682.

4.1 0.05mol/L Tris buffer solution (pH 8.0): Phenol containing mass fraction 0.05%

Dissolve 6.057g trihydroxymethyl aminomethane and 0.5g phenol in a 1000ml volumetric flask, which is filled with about 500ml distilled water, and dilute them with distilled water to volume. The solution may be regulated with 6mol/L hydrochloric acid to pH 8.0.

4.2 Color developing agent for enzyme reaction

In a 50ml volumetric flask, dissolve 7.81ml choline oxidase (12.8U/mg), 1.31mg peroxydase (190U/mg), 7.5mg 4- amino-antipyrine and 0.02mg phosphatidase and dilute them with Tris buffer solution (4.1) to volume.

4.3 Hydrochloric acid: c (HCL) is 3mol/L. Suck 125ml concentrated hydrochloric acid and dilute it with water to 500ml.

4.4 Hydrochloric acid: c (HCL) is 1mol/L. Suck 42ml concentrated hydrochloric acid and dilute it with water to 500ml.

4.5 Sodium hydroxide solution: Mass fraction is 60%.

4.6 Standard solution: 250ug/ml hydroxide containing choline

Dissolve 523mg choline bitartrate ($C_5H_{14}NO \bullet C_4H_5O_6$) in a 100ml volumetric flask and dilute it with distilled water to volume. Suck 10ml of this solution with a volumetric pipette into a 100ml volumetric flask and dilute it with distilled water to volume.

5. Instruments and devices

5.1 Electronic balance: Sensitive to 0.01g of weight

5.2 Analytical balance: Sensitive to 0.1mg of weight

5.3 Circumfluence extractor: 250ml flat-bottom and ground flask and circumfluence device

5.4 Spectrophotometer

6. Analyzing steps

6.1 Preparation of test sample

Homogenize the test samples by blending or grinding and weigh and take the test samples accurate to 0.01g so that the samples' choline content is approximately 1mg to 10mg (calculated in hydroxide).

6.1.1 Solid test samples

Weigh and take 5g well blended test samples into a 250ml flat bottom ground flask and add in 30ml hydrochloric acid (4.4) and blend it.

6.1.2 Liquid test samples

Weigh and take 20g well blended test samples into a 250ml flat bottom ground flask and add in 10ml hydrochloric acid (4.3) and blend it.

6.1.3 Hydrolysis

Connect the container filled with the test samples to the reflux device, warm it in 70℃ water bath for 5 hours, during which stir it with a magnetic stirring apparatus, then cool it, and regulate its pH value to 3.4 ~ 3.6 using sodium hydroxide solution (4.5). Cool it again if necessary, and transfer it into a 50ml volumetric flask, and then dilute it with distilled water to volume.

6.1.4 Filtration

Filter the hydrolyzed liquor (6.1.3). The filtered liquor should be clear. But if it is not clear, filter it again with a 0.45um filter membrane. If clear filtered liquor can't be got due to the nature of test sample or due to difficulty of filtering, dilute its pH value to 4.0 ~ 4.5.

6.2 Determination

6.2.1 Sample determination

Prepare 3 test tubes (A, B, C) for each of the test samples and add in the reagents as stated in Table 1.

Table 1

Reagent ml	Test tube A Reagent blank	Test tube B Filtered liquor blank	Test tube C Test sample
Filtered liquor to be analyzed	-----	0.100	0.100
Distilled water	0.100	3.00	-----
Color former	3.00	-----	3.00

Cover the test tubes with protective sealing films, blend their contents, and put the test tubes into 37℃ water baths for reaction for 10 minutes.

6.2.2 Making standard curve

Transfer 2ml, 4ml, 6ml and 8ml standard solution (4.6) with a graduated suction tube to four 10ml volumetric flasks respectively and dilute the solution with distilled water to volume.

Prepare 6 test tubes, of which one is used for reagent blank (A), and the other five are numbered 1 to 5 and used for the standard solution and the four diluting degrees of the standard solution respectively, and add in reagents as stated in Table 2.

Table 2

Reagent ml	Test tube A	Test tube 1	Test tube 2	Test tube 3	Test tube 4	Test tube 5
Diluting degree 1 (50ug/ml)	—	0.100	—	—	—	—
Diluting degree 2 (100ug/ml)	—	—	0.100	—	—	—
Diluting degree 3	—	—	—	0.100	—	—

(150ug/ml)						
Diluting degree 4 (200ug/ml)	—	—	—	—	0.100	—
Standard solution (250ug/ml)	—	—	—	—	—	0.100
Distilled water	0.100	—	—	—	—	—
Color former	3	3	3	3	3	3

Cover the test tubes with protective sealing films, blend their contents, and put the test tubes into 37℃ water baths for reaction for 10 minutes.

6.2.3 Colorimetric determination

Take out the test sample and the standard solution series from water baths and cool them to room temperature. Regulate the wave length of spectrophotometer to 505nm and determine the light absorption value using distilled water as blank. Draw a working curve using the concentration of choline standard solution as the horizontal axis and the light absorption value of the standard solution as the vertical axis, and find out the concentration of choline in the hydrolysate of test samples on the working curve.

7. Result calculation and presentation

7.1 Calculation of net light absorption value

Generally, a reagent prepared most lately shows a mild color and due to hydrolytic action, the hydrolysate is not colorless. To eliminate these interfering factors, respective blank values (test tubes A and B) should be deducted from the total light absorption value.

$$A = A_{tot} - A_{b1} - A_{ex} \quad \text{(1)}$$

Where:

A — Net light absorption value of test sample

A_{tot} — Total light absorption value (test tube C)

A_{b1} — Light absorption value of reagent (test tube A); and

A_{ex} — Light value of extract (test tube B)

A_{b1} and A_{ex} shall be no greater than 20% of total light absorption value, and for the standard curve, A_{ex} =0

7.2 Calculation of choline content

Find out the position of the net light absorption value on the standard curve and write down the concentration c. Choline content (*X*) expressed with the unit (m/100g), i.e. mg number of choline hydroxide per 100g test sample, is calculated as follows:

$$X = \frac{c \times V \times 100}{m \times 1000}$$

Where:

X — Choline content in test sample, mg/100g;

c — Choline concentration found out on the standard curve, ug/100g;

V — Volume to which the hydrolysate is diluted (generally 50ml), ml; and

m — Mass of test sample, g.

Take the mean arithmetical value of the two separate determined results as the final determined result, with one figure after decimal point.

8. Precision

Absolute difference between two separate determined results under repetitive conditions should be no greater than 8% of the mean arithmetical value.

The Second Method: Reinecke's Salt Spectrophotometric Method

9. Principle

Choline in test samples is extracted by hydrolyzing with mixed barium hydroxide – methanol – chloroform solution, purified by chromatography with Florisil, reacts with Reinecke's salt solution on the chromatography column to form pink choline Reinecke's salt, which is eluted with acetone and tested for absorption value at 526nm. Within a certain range of concentration, the shade of color of choline Reinecke's salt is positively proportional to its content.

10. Reagents and materials

Unless otherwise provided, in this method, all reagents are analytically pure and all water is third level water as provided in GB/T 6682.

10.1 Florisil: 60 – 120 mesh, 650℃ activated

10.2 Methanol

10.3 Acetone

10.4 Glacial acetic acid

10.5 Methyl acetate

10.6 Glacial acetic acid – methanol solution (volume ratio: 1:10)

10.7 Saturated barium hydroxide – methanol – chloroform solution: Weigh and take 5g anhydrous barium hydroxide and dissolve it in 100ml methanol, stir the solution for 5 to 10 minutes, and then add in 10ml chloroform, and blend the solution properly.

10.8 Ammonium reineckate solution: Weigh and take 2.5g ammonium reineckate (accurate to 0.1g), put it in 100ml, stir the solution for 10 minutes, and filter out redundant salt. Prepare the solution for use in the current day.

10.9 Choline standard solution: Choline bitartrate solution ($C_5H_{14}NO \cdot C_4H_5O_6$) 1g/L

Weigh and take 1.0000g choline bitartrate (accurate to 2mg), which has been dried in a dryer for more than 2 hours beforehand, and dissolve it with distilled water in a 100ml volumetric flask to volume.

11. Instruments and devices

11.1 Electronic balance: Sensitive to 0.1g of weight.

11.2 Analytical balance: Sensitive to 0.1mg of weight.

11.3 Circumfluence device: 250ml ground triangular flask and circumfluence device

11.4 Water bath kettle

11.5 Chromatographic column: Glass column which is 10cm in length and 1cm in internal diameter, with 50ml cup mouth

11.6 Spectrophotometer

12. Analyzing steps

12.1 Preparation of test samples

Weigh and take 10g solid test sample and 20g properly blended liquid test sample (accurate to 0.01g), put the samples into ground triangular flasks, and add in 50ml barium hydroxide – methanol – chloroform extract (10.7). Blend the content properly, make it connected to the reflux device, and extract it by hydrolyzing in 79℃±2℃ water bath for 4 hours. Agitate it every 30 minutes to prevent the test sample from clotting. After completion of hydrolyzed extraction, take out the triangular flask, cool it to room temperature, and filter its content. Wash the filter residue with glacial acetic acid – methanol mixture (10.6) for 3 to 4 times, collect all washed liquor into a 100ml volumetric flask, and dilute the washed liquor with methanol to volume and blend it properly.

12.2 Purifying and color developing

12.2.1 Preparation of chromatographic column

Connect the chromatographic column’s bottom mouth and the dropper head, plug up the chromatographic column’s bottom with certain amount of absorbent cotton, pour Florisil into the column for about 5cm in depth, and add in methanol solution to make the chromatographic column fully wetted.

12.2.2 Purification

After methanol solution has fully entered into the chromatographic column, add in 10ml test sample hydrolysate (12.1). After sample hydrolystate has fully entered into the column bed, clean the chromatographic column using 5ml and 10ml methanol (10.2) and 20ml methyl acetate (10.5) respectively, then add in 5ml reinecke's salt. After the reinecke's salt has fully entered into the column bed, clean out redundant reinecke's salt with a suitable amount of glacial acetic acid (10.4), until the original white of Florisil is shown at areas without choline reinecke's salt on the

chromatographic column. Elute pink choline reinecke's salt with acetone, collect it in a 10ml volumetric flask, and dilute it with acetone to volume (the eluent should go through 0.45um filter film, if it is turbid).

12.2.3 Drawing standard curve

Suck and pour 1.0ml, 2.0ml, 3.0ml, 4.0ml and 5.0ml choline standard solution into chromatographic columns and operate as per the test sample purifying steps (12.2.2): collect pink choline reinecke's salt acetone solution and dilute it with acetone to 10ml.

12.3 Color comparison

With acetone as the reference color, determine the light absorption value of the test sample and the standard solution series at 526nm. Draw a standard curve across the dots, using the content of choline bitartrate as the horizontal axis (m_x) and the light absorption value as the vertical axis. Find out the content of choline bitartrate in 10ml test sample hydrolysate.

13. Analytical result calculation and presentation

Choline in test sample is measured in choline hydroxide, expressed with gram per hundred grams (g/100), calculated as follows:

$$X = \frac{m_x}{\frac{m}{100} \times V} \times 100 \times 0.474 \quad \cdots\cdots (2)$$

Where:

X — Content of choline hydroxide in test sample, mg/100g;

m — Mass of test sample, g;

V — Number of milliliters of test sample hydrolysate sucked in chromatography ml;

m_x — Content of choline bitartrate found out on the standard curve, mg; and

0.474 — Coefficient at which choline bitartrate is transformed to choline oxide

Take the mean arithmetical value of the two separate determined results as the final determined result, with one figure after decimal point.

14. Precision

Absolute difference between two separate determined results under repetitive conditions should be no greater than 8% of the mean arithmetical value.

The Third Method: Ion Chromatographic Method

15. Principle

The test sample is hydrolyzed by hydrochloric acid to generate free choline, which is separated by capture exchange column, tested by electric conductivity detector, and quantified by external reference method.

16. Reagents and materials

Unless otherwise provided, in this method, all reagents are analytically pure and all water is first level water as provided in GB/T 6682.

16.1 Acetone: Chromatographically pure

16.2 Tartaric acid: Analytically pure

16.3 Concentrated hydrochloric acid: Analytically pure

16.4 Choline chloride ($C_5H_{14}NCIO$): Purity no less than 99.0%

16.5 Hydrochloric acid solution (1 mol/L): Measure and take 90ml hydrochloric acid and dilute it with a certain amount of water to 1000ml.

16.6 Choline standard solution (1mg/ml): Dry choline chloride in 105℃ for constant weight, weigh and take 0.139g dried choline chloride (accurate to 0.1mg) and put it into a 100ml volumetric flask, dissolve it with water and dilute it to volume to prepare 1mg/ml choline standard solution (measured in choline), which should be prepared for immediate use.

16.7 Choline standard series working solution: Accurately suck 1ml, 2ml, 5ml, 10ml and 20ml choline standard solution respectively and put them in 100ml volumetric flasks and dilute them with water to volume. As such, the concentration of this standard series is 10mg/L, 20mg/L, 50mg/L, 100mg/L and 200mg/L respectively.

16.8 Ion chromatographic leaching stock solution: Weigh and take 6g tartaric acid (accurate to 0.1g) and put it in a 1000ml volumetric flask, dissolve it with water, add in 175ml acetone, and dilute it to volume. This solution can be kept for 1 month in 4℃.

16.9 Ion chromatographic leacheate (0.004mol/L acetone tartrate water solution): Take 100ml iron chromatographic leaching stock solution, put it into a 1000ml volumetric flask, dilute it with water to volume, and filter it using 0.45μm micro-porous filter film.

16.10 Microporous filtering film: Grain diameter 0.45μm, aqueous phase

16.11 Conical flask with cork

17. Instruments and devices

17.1 Ion chromatograph: With electric conductivity detector

17.2 Thermostatic water bath kettle

17.3 Analytical balance: Sensitive to 0.1mg of weight

18. Analyzing steps

18.1 Treatment of samples

Weigh and take 5g test sample (accurate to 0.01g), blend it properly and put it in a 50ml conical flask with stopper, add in 30ml hydrochloric acid solution, shake it properly, and make it hydrolyzed in 70℃ thermostatic water bath kettle for 3 hours (jolt it every 1 hour to avoid milk powder from sticking to wall), and then take it out and cool it to room temperature. Transfer the hydrolysate to a 50ml volumetric flask, dilute it with water to volume, filter the diluted liquor with filter paper, and filter the filtrate with 0.45μm filter film for future determination.

18.2 Ion chromatographic reference conditions

Chromatographic column: Metrosep C4 100mm X 4.0mm, grain diameter 5μm or equivalent;

Leacheate: 0.004mol/L acetone tartrate water solution;

Flow speed: 0.9ml/min;

Sample size: 20μL;

Detector: Electric conductivity detector

18.3 Determination

Introduce samples of standard working solution series and test solution respectively, and draw a standard working curve, using concentration as the horizontal axis and the peak area as the vertical axis. Quantify the samples using the standard working curve. The response value of choline in the test solution should be within the linear range of the standard curve, but if it is outside the linear range, the test solution should be diluted for sample introduction and analysis. See Appendix A for chromatogram map of the standard solution and test solution under the said chromatographic condition.

18.4 Parallel test

A same sample is determined under parallel test as per the above-stated steps.

19. Result calculation

Ion chromatographic determination result can be calculated automatically by the instrumented working station or as per formula (3):

$$X = \frac{c \times V}{m} \qquad \text{.....(3)}$$

Where:

X — Choline content in the test sample, expressed in a unit of milligram per kilogram (mg/kg);

c — Concentration of solution of ingredient under test found out on the standard working curve, expressed in a unit of milligram per liter (mg/L);

V — Final volume of the test sample, expressed in a unit of milliliter (ml); and

m — Mass of the test sample, expressed in a unit of gram (g)

20. Precision

In this standard, the precision data are identified in accordance with GB/T 6379.2 and the repeatability and reproducibility is calculated at 95% confidence.

20.1 Repeatability

Under repeatable conditions, the absolute difference between two separately determined results should be no greater than the repeatability limit (r). See Table 1 for the repeatability equation for this standard.

20.2 Reproducibility

Under reproducible conditions, the absolute difference between two separately determined results should be no greater than the reproducibility limit (*R*). See Table 1 for the reproducibility equation for this standard.

Table 1 Repeatability and Reproducibility Equations

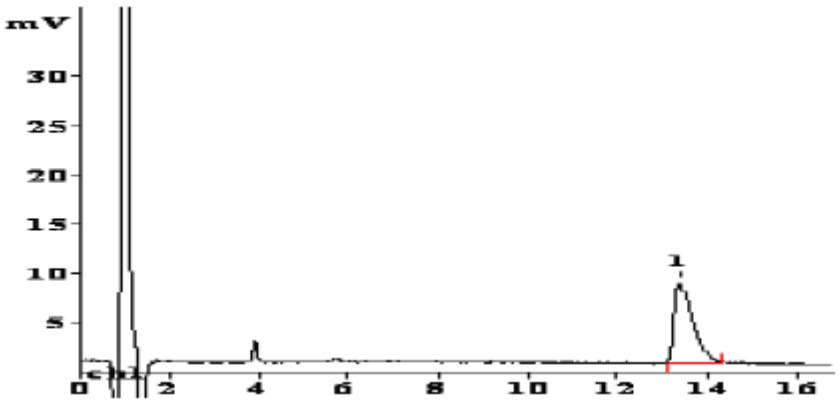
Name	repeatability limit *r*	reproducibility limit *R*
Choline	$\lg r=-0.594+0.461\ \lg m$	$\lg R=-1.211+0.903\ \lg m$
M is the mean of the two separately determined results, expressed in a unit of milligram per kilogram (mg/kg)		

Appendix A

(Informative annex)

Chromatogram map for Determination of Choline Content in Formulated Milk Powder for Infants and Young Children

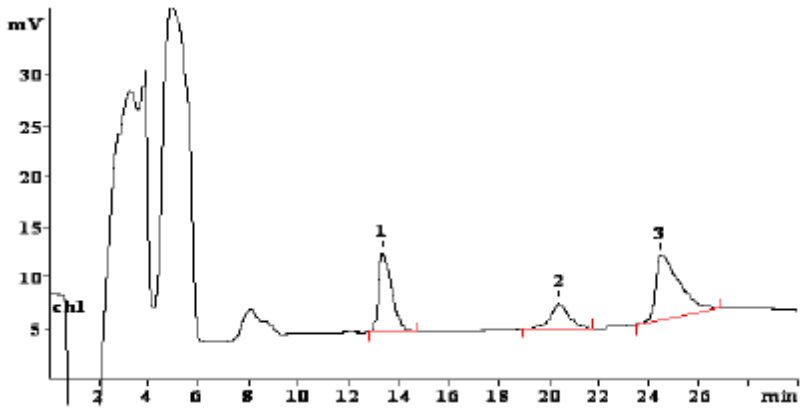
See Figure A1 for ion chromatogram map of choline standard substance



1 – Choline

A.1: Ion chromatogram map of choline standard substance

See Figure A.2 for ion chromatogram map of formulated milk powder for infants and young children



1 – Choline, 2 – Magnesium, 3 – Calcium

A.2: Ion chromatogram map of formulated milk powder for infants and young children